

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
20 February 2003 (20.02.2003)

PCT

(10) International Publication Number
WO 03/013596 A1

(51) International Patent Classification: A61K 39/04,
A61P 31/04, 31/06

(21) International Application Number: PCT/NZ02/00152

(22) International Filing Date: 7 August 2002 (07.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
513418 7 August 2001 (07.08.2001) NZ

(71) Applicants (for all designated States except US):
MASSEY UNIVERSITY [NZ/NZ]; Fitzherbert West,
Palmerston North (NZ). THE NEW ZEALAND MEAT
BOARD [NZ/NZ]; Level 10, Wool House, 10 Brandon
Street, Wellington (NZ). WOOL PRODUCTION TECH-
NOLOGIES LTD [NZ/NZ]; 13th Floor, Wool House, Cnr
of Brandon & Featherston Street, Wellington (NZ).

(72) Inventors; and

(75) Inventors/Applicants (for US only): MURRAY, Alan
[NZ/NZ]; 6 Williams Terrace, Palmerston North (NZ).
DUPONT, Christine [CA/NZ]; 5 Luton Street, Palmer-
ston North (NZ). RAE, Jeremy, Lawrence [NZ/NZ]; 16
Anchorage Grove, Mangatapu, Tauranga (NZ).

(74) Agents: ADAMS, Matthew, D et al.; A J Park, 6th Floor
Huddart Parker Building, Post Office Square, PO Box 949,
Wellington 6015 (NZ).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, EC, EE, ES, FI, GB, GD, GE, GH, GM,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK,
LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,
MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI,
SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC,
VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK,
TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

WO 03/013596 A1

(54) Title: VACCINE

(57) Abstract: The invention provides a vaccine comprising secreted protein derived from *Mycobacterium avium* subsp *paratuberculosis* (M. ptb) substantially free of whole organisms of that species either dead or alive. The secreted protein may be obtained from a culture of M. ptb with the microorganisms being removed by centrifugation and subsequent filtration. The vaccine may be used for vaccination against Johne's disease.

BEST AVAILABLE COPY

VACCINE

TECHNICAL FIELD

This invention relates to vaccines comprising proteins from *Mycobacterium avium*
5 subsp *paratuberculosis*.

BACKGROUND ART

Johne's disease (paratuberculosis) is a chronic, contagious infection with the acid-fast-staining bacillus *Mycobacterium avium* subsp *paratuberculosis* (M.ptb). The disease
10 affects ruminants and is characterised by emaciation and intermittent diarrhoea or softening of faeces. Johne's disease is a major disease of cattle, sheep, goats, deer, and camels (Beeman et al, The Compendium 11,1415 (1989)).

The currently favoured treatment is with a living vaccine (Neoparasec, Merial). This
15 vaccine contains live organisms of the Weybridge strain, an attenuated strain of M.ptb. Killed vaccines are also known.

The existing vaccines have two disadvantages. Carcasses of animals treated with the vaccines contain whole organisms which are not readily distinguishable from
20 tuberculosis organisms. In addition both types of vaccines leave injection site lesions which can be easily confused with tuberculosis lesions.

An object of the present invention is to prepare a vaccine against Johne's disease in which the above disadvantages are either not present or are reduced.

DISCLOSURE OF THE INVENTION

In one aspect the invention provides a vaccine comprising secreted protein derived from *Mycobacterium avium* subsp *paratuberculosis* (M. ptb) which is substantially free of whole organisms of that species either dead or alive. Preferably there are no organisms
5 of M. ptb. The vaccine of the invention may be used for treating Johne's disease.

The term "substantially free" indicates that the number of live or dead organisms is too few to have significance in the vaccination process (for example fewer than 1000/ml).

10 Preferably the secreted proteins are obtained from a culture of M. ptb.

Preferably the microorganisms are of an attenuated strain.

Preferably the strain is the Weybridge vaccine strain.

15

Preferably the vaccine additionally comprises an adjuvant.

Preferably the vaccine comprises a serum albumin, more preferably a heterologous serum albumin. For sheep the currently preferred albumin is bovine serum albumin.

20 For cows use of ovine serum albumin is preferred.

Preferably where the secreted proteins are obtained from a microorganism culture, the microorganisms are removed by centrifugation and subsequently filtered to remove remaining bacteria.

25

Preferably the secreted proteins are concentrated using ultrafiltration.

The term "secreted proteins" herein refers to proteins present in the supernatant after centrifugation for 10 min at 10,000g of a culture of *Mycobacterium avium* subsp *paratuberculosis* organisms. In addition to exported proteins the supernatant includes proteins which have sloughed off the microorganisms or are present in the culture as a result of other causes.

In a second aspect the invention provides a vaccine against Johne's disease comprising a supernatant of a *Mycobacterium avium* subsp *paratuberculosis* culture which does not contain whole organisms of that species, either dead or alive.

In a third aspect the invention provides a use of a supernatant as defined in the second aspect for the preparation of a medicament for vaccinating an animal against *Mycobacterium avium* subsp *paratuberculosis*.

In a fourth aspect the invention provides a method for vaccinating an animal against *Mycobacterium avium* subsp *paratuberculosis* comprising administering a vaccine of the invention to the animal. Preferably the vaccination is against Johne's disease. Animals for which the method of the invention is particularly useful include ruminants, especially sheep.

DESCRIPTION OF THE DRAWINGS

Figure 1 is graph showing isocitrate dehydrogenase (ICD) in concentrated CF and in cell sonicates plotted against OD600.

Figure 2 is a copy of an SDS-PAGE gel of growth medium (lane 2) and CF (lane 3).

Figure 3 is a graph showing gamma interferon produced in the blood samples of six
5 vaccinated animals and three control animals in response to Johnin PPD and candidate
vaccine antigens.

Figure 4 is a graph showing the interferon produced in blood samples taken from
unvaccinated animals and animals vaccinated with Neoprasec, Culture Filtrate (CF)
10 plus Neoprasec Adjuvant (NPA), and media plus NPA, in response to Avium PPD and
CF.

Figure 5 is a graph showing the interferon produced in blood samples taken from
unvaccinated animals and animals vaccinated with Neoprasec and CF plus Neoprasec
15 adjuvant, in response to Avian PPD, Johnin PPD and CF antigen.

Figure 6 is a graph of mean interferon gamma response to Avian PPD against the
number of months after vaccination.

20 Figure 7 is a graph of mean interferon gamma response to CF against the number of
months after vaccination.

EXAMPLES

The following Examples further illustrate practice of the invention.

EXAMPLE I

Studies were performed with three-month old male neutered Romney lambs obtained from Massey Agricultural Services, Palmerston North, New Zealand. The animals were kept on farming blocks with open grazing and water *ad libitum*. The sheep used in this study were selected on the basis of negative reactivity with Johnin PPD mycobacterial antigen, as measured by the BOVIGAM (CSL) whole blood IFN- γ assay kit.

CF Antigen

Candidate Johnne's vaccine antigen Culture Filtrate (CF), which contains *M. ptb* secreted proteins was prepared from liquid media cultures of *M. ptb* Weybridge vaccine strain (Neoparasec). The cultures were grown to early mid-log phase and the cells removed by centrifugation. The resultant supernatants (containing proteins secreted by *M. ptb*) were filtered to remove remaining bacteria and concentrated approximately 200-fold using ultrafiltration.

15

CF and Media-only (M) control samples were quantified using a protein assay and diluted appropriately in Phosphate-Buffered Saline (PBS). It is estimated that 5-10% of total CF is *M. ptb* secreted protein, the remainder being bovine serum albumin (BSA).

20 *M. ptb* cultures and quantification of ICD

M. ptb Weybridge reference strain 316F was obtained from Rhone-Merieux in freeze-dried form and rehydrated in Middlebrook 7H9 broth (Difco), supplemented with 1 mg/L Mycobactin J (Allied Monitor), 0.2% Bactoglycerol (Difco), 1:100 (v/v) Middlebrook ADC Enrichment (Becton Dickenson) and dextrose to a final concentration of 2g/L. *M. ptb* was propagated on 7H10 (Difco) agar slopes

25

supplemented with Middlebrook OADC Enrichment as recommended by the manufacturer (Becton Dickenson) and Mycobactin J added as above. Liquid cultures (7H9) were grown at 37°C with vigorous shaking.

- 5 The growth of *M. ptb* in liquid medium was monitored by taking Optical Density (OD) readings at 600nm, at regular intervals for a period of three weeks. The degree of lysis in the cultures was determined by quantification of isocitrate dehydrogenase (ICD), a cytoplasmic marker, in the 200-fold concentrated supernatants (see below). *M. ptb* sonicates were used as positive controls. ICD activity was determined using the ICD
10 diagnostic kit for quantitative determination (Sigma), with changes in absorbance at 340 nm corresponding to the reduction of NADP to NADPH. Results are expressed as international units, which are equal to micromoles of NADPH formed per minute at 25°C. Very little lysis was observed in the preparation of the cell filtrates (see Figure 1).

15

Preparation of M.ptb culture filtrate (CF) protein

- For preparation of culture filtrate, 5 ml of *M. ptb* starter culture was grown to late log phase, inoculated 1:100 into fresh media and grown to mid-log phase (approximately 3 weeks) at 37°C with vigorous shaking. The cells were removed by centrifugation at
20 approximately 10,000x g and the culture supernatant was passed through a 0.22 µm filter and concentrated by ultrafiltration using an Amicon apparatus containing a 3,000 MW cut-off membrane. The concentrated material was buffer-exchanged by repeated dilutions with PBS buffer and re-concentrated (approx. 1000-fold dilution). For the final concentration step the CF was centrifuged at 3000x g in a Centriplus (Amicon)
25 apparatus with a 3,000 MW cut-off. The resulting 200-fold concentrated CF was stored

at -20°C until required. Protein concentrations were determined using Bio-Rad Protein Assay reagent with bovine serum albumin (BSA) as standard. Control (liquid media only samples) were prepared by filtering and concentrating media as described above.

- 5 Figure 2 is a copy of an SDS-PAGE gel showing a comparison of the CF proteins (lane 3) with the medium proteins. Major M.ptb bands have molecular weights of approximately 27 and 40KD.

Experimental

- 10 A flock of twelve lambs (three months of age) were selected to test the immunogenicity of the CF antigens. Six sheep were vaccinated with the commercial Johnne's vaccine Neoparasac (as per the manufacturer's instructions). The remaining sheep were kept as non-vaccinated controls. At monthly intervals blood was obtained from the sheep and tested against candidate vaccine antigens using a BOVIGAM gamma interferon assay
- 15 kit. Production of gamma interferon by lymphocytes in whole blood is a measure of cell-mediated (protective) immune response to a particular antigen. Each antigen was incubated with 1 ml of blood overnight, then the supernatant was analysed by BOVIGAM ELISA to determine the levels of gamma interferon produced.

- 20 Six months after vaccination, CF (containing Neoparasac secreted proteins) was included in the monthly bleeds in the following amounts (per 1ml of sheep blood): H = High=150 λ g, M = Medium=75 λ g and L = Low=25 λ g. Media-only (containing equivalent amounts of media protein) readings were subtracted from the CF readings. In most instances, readings for negative controls did not exceed OD 0.1. Johnin PPD
- 25 (positive control) was 12.5 λ g for all animals. Johnin PPD is an extract from whole M.

ptb organisms. PBS served as a negative control for PPD and was subtracted from the PPD readings shown. ODs shown in Figure 3 are means from triplicate readings (triplicate blood/antigen incubations).

- 5 The response to CF was consistently higher in vaccinates than in non-vaccinated control animals. This effect was observed throughout the samples collected in the period 6-10 months. Within animals, response to culture filtrates was shown to be significantly above responses to media controls within vaccinates in almost all cases. Occasionally controls showed significant reactions to Johnin and CF (e.g. 134c, Figure 3) but this was
10 not consistently observed over different assays.

To provide further evidence that the responses seen resulted from protein antigens in the CF, and not other (non-protein) components CF, protein was precipitated using ammonium sulphate (A.S.) and was included in the 7 month assay. There was still a
15 significant reaction to this fraction, whereas the remaining non-protein supernatant (not shown) gave a very low response. This result indicates that the protein fraction of CF is indeed responsible for the stimulation observed.

Conclusions

- 20 At least part of the "protective type" immune response following Neoparasec vaccination of sheep appears to be due to proteins secreted by the vaccine strain.

EXAMPLE II

Animals were randomly assigned to four different treatment groups. Group 1 received no vaccination. Group 2 received Neoprasec according to the manufacturer's instructions. Group 3 received CF plus the Neoprasec adjuvant. Group 4 received
5 medium plus Neoprasec adjuvant.

After 1, 2, 3, 4 and 6 months blood was collected from each of the animals. These monthly blood samples were tested for gamma interferon production by lymphocytes as a measure of cell-medium (protective immune response) to Avium PPD, Johnin PPD
10 and CF M. The result showed that immunisation with Neoprasec gave a high response in all the in vitro tests for all the animals. The animals which were unvaccinated or vaccinated with media plus NPA gave samples which showed very little interferon production in response to PPD or CF M. An intermediate response was shown in the
15 animals which had been vaccinated with CF plus NPA. The results for the 1 month assay are shown in Figure 4. Similar results were shown in the blood samples over the following four months (not shown). Figure 5 shows that even 6 months after vaccination, there is an interferon production response to Johnin PPD and CF. The time course of the response to Avian PPD and CF is shown in Figures 6 and 7 respectively.

20 The injection site lesions were scored at 0.5, 1, 2, 3 and 4 months post injection. At the 0.5 month examination the CF plus NPA group had the highest mean score of the four groups (including those treated with the live vaccine Neoprasec). At all subsequent examinations the mean scores for the CF plus NPA group had fallen below those for the Neoprasec group, but remained higher than for the group receiving media + NPA and
25 the unvaccinated group.

At 2 months post-vaccination, antibody levels were measured using Paracheck (Johne's Absorbed EIA for the determination of paratuberculosis, CSL Ltd). The antibody levels were significantly higher in both the Neoparasec-vaccinated and CF-vaccinated groups
5 than in the naive animals.

The above Examples are illustrations of practice of the invention. It will be appreciated by those skilled in the art that the invention can be carried out with numerous modifications and variations. For example the vaccinations may use a variety of
10 different adjuvants, the strain of bacterium used to prepare the secreted proteins may be varied and the secreted proteins may be fractionated.

CLAIMS

1. A vaccine comprising secreted protein derived from *Mycobacterium avium* subsp *paratuberculosis* (M. ptb) which is substantially free of whole organisms of that species either dead or alive.
2. A vaccine as claimed in claim 1 wherein the secreted proteins are obtained from a culture of M. ptb.
3. A vaccine as claimed in claim 1 or claim 2 wherein the microorganisms are of an attenuated strain.
4. A vaccine as claimed in claim 3 wherein the strain is the Weybridge vaccine strain.
5. A vaccine as claimed in any one of claims 1 to 4 which comprises an adjuvant.
6. A vaccine of any one of claims 1 to 5 which comprises a serum albumin.
7. A vaccine as claimed in claim 2 wherein the secreted proteins are obtained from a microorganism culture; the microorganisms being removed by centrifugation and subsequent filtration.

8. A vaccine against Johne's disease comprising a supernatant of a *Mycobacterium avium* subsp *paratuberculosis* culture which does not contain whole organisms of that species, either dead or alive.

5 9. A method for vaccinating an animal against *Mycobacterium avium* subsp *paratuberculosis* comprising administering a vaccine as claimed in any one of claims 1 to 8 to the animal.

10. A method as claimed in claim 9 wherein the animal is a ruminant.

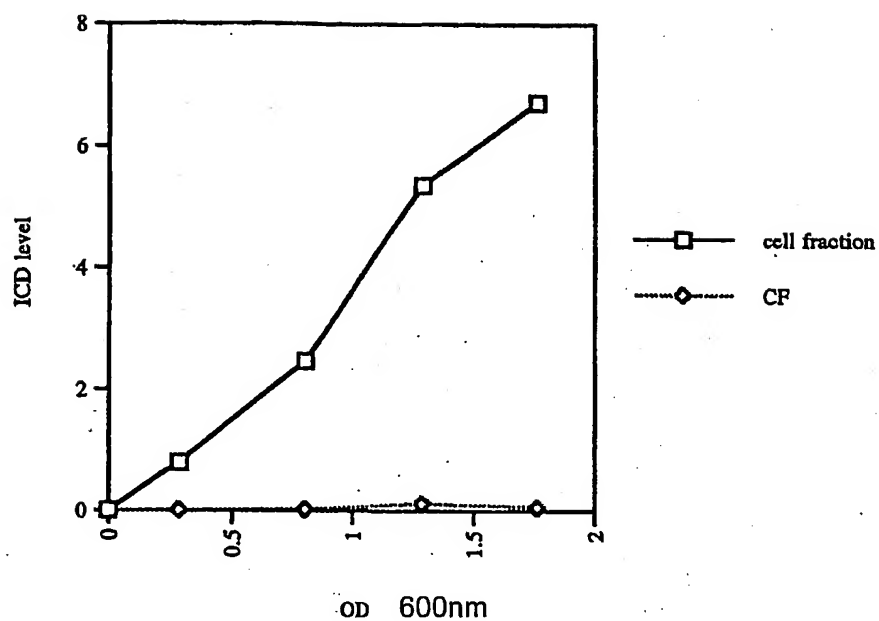
10

11. A method as claimed in claim 10 wherein the ruminant is a sheep.

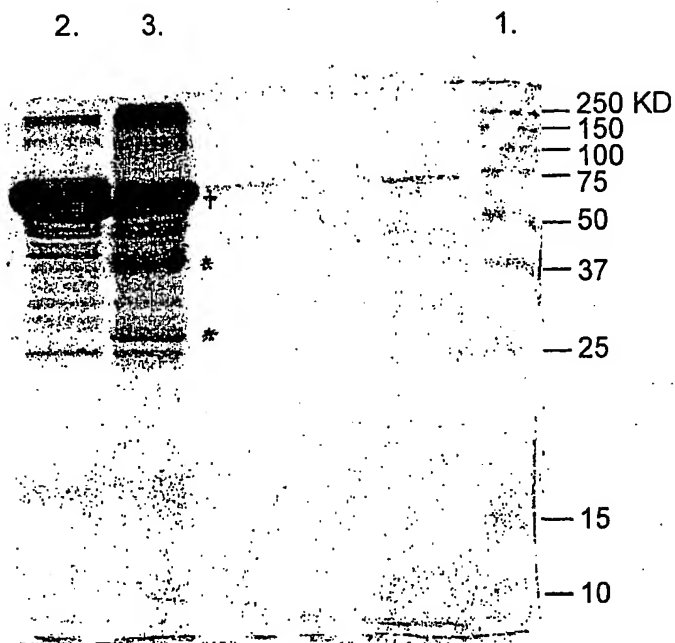
12. Use of the supernatant defined in claim 7 for the preparation of a medicament for vaccinating an animal against *Mycobacterium avium* subsp *paratuberculosis*.

15

1/7

**FIGURE 1**

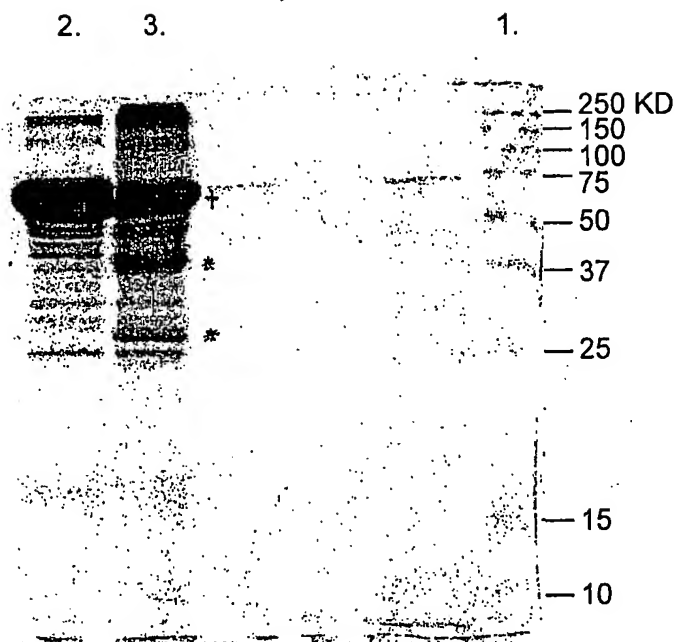
2/7

**Lane**

1. Biorad Precision protein Standard
2. 20µg growth medium
3. 20µg of M.ptb culture filtrate (CF)
- * Major M.ptb protein bands
- + Albumin band

FIGURE 2

2/7

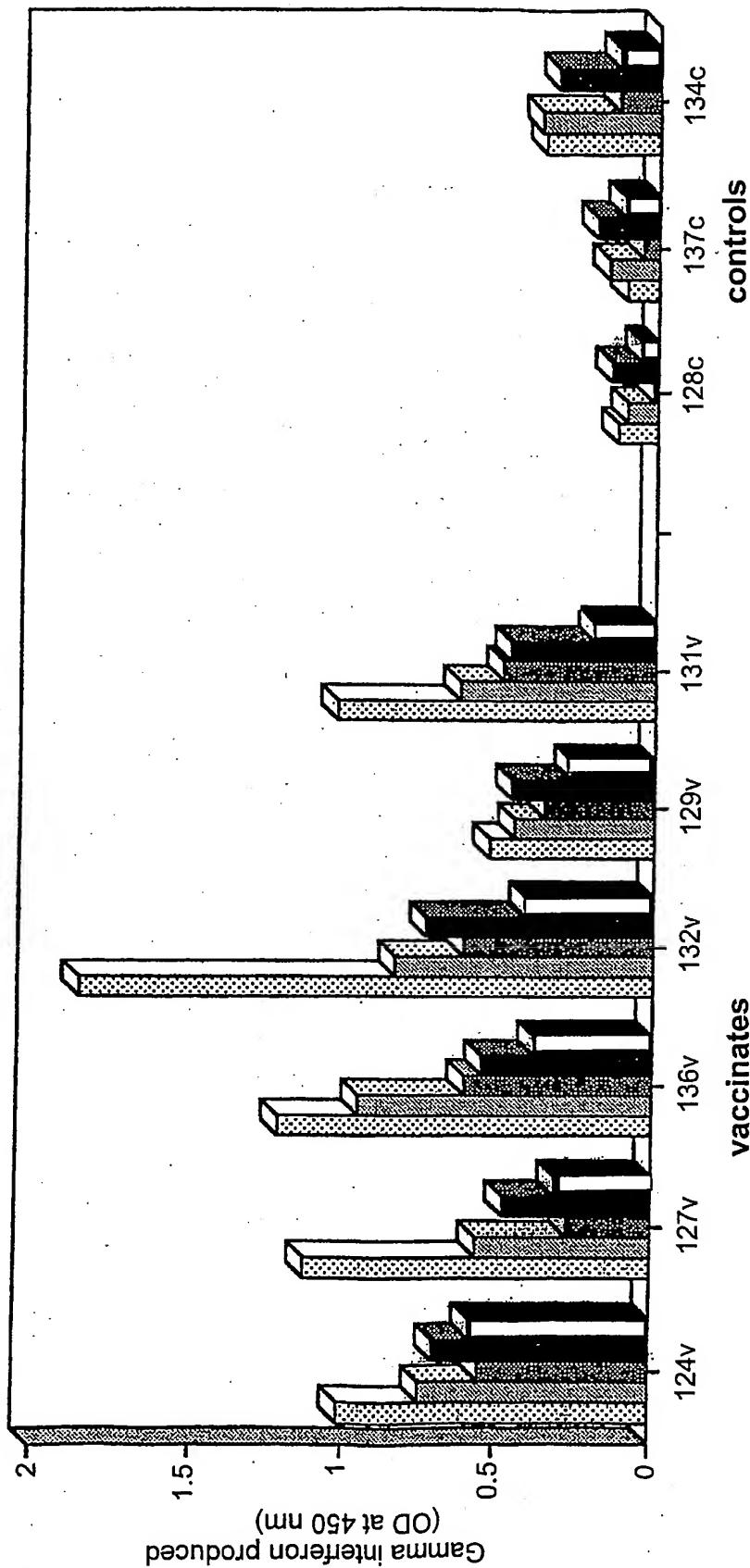


- Lane**
1. Biorad Precision protein Standard
 2. 20µg growth medium
 3. 20µg of M.ptb culture filtrate (CF)
- * Major M.ptb protein bands
+ Albumin band

FIGURE 2

3/7

Bleed 9 Bovigam 11/07/00 - 7 months post -vacc



Animal

FIGURE 3

Bleed 2 13/2/01: BOVIGAM of CF and Neoprasec vaccinated sheep - PBS/Media

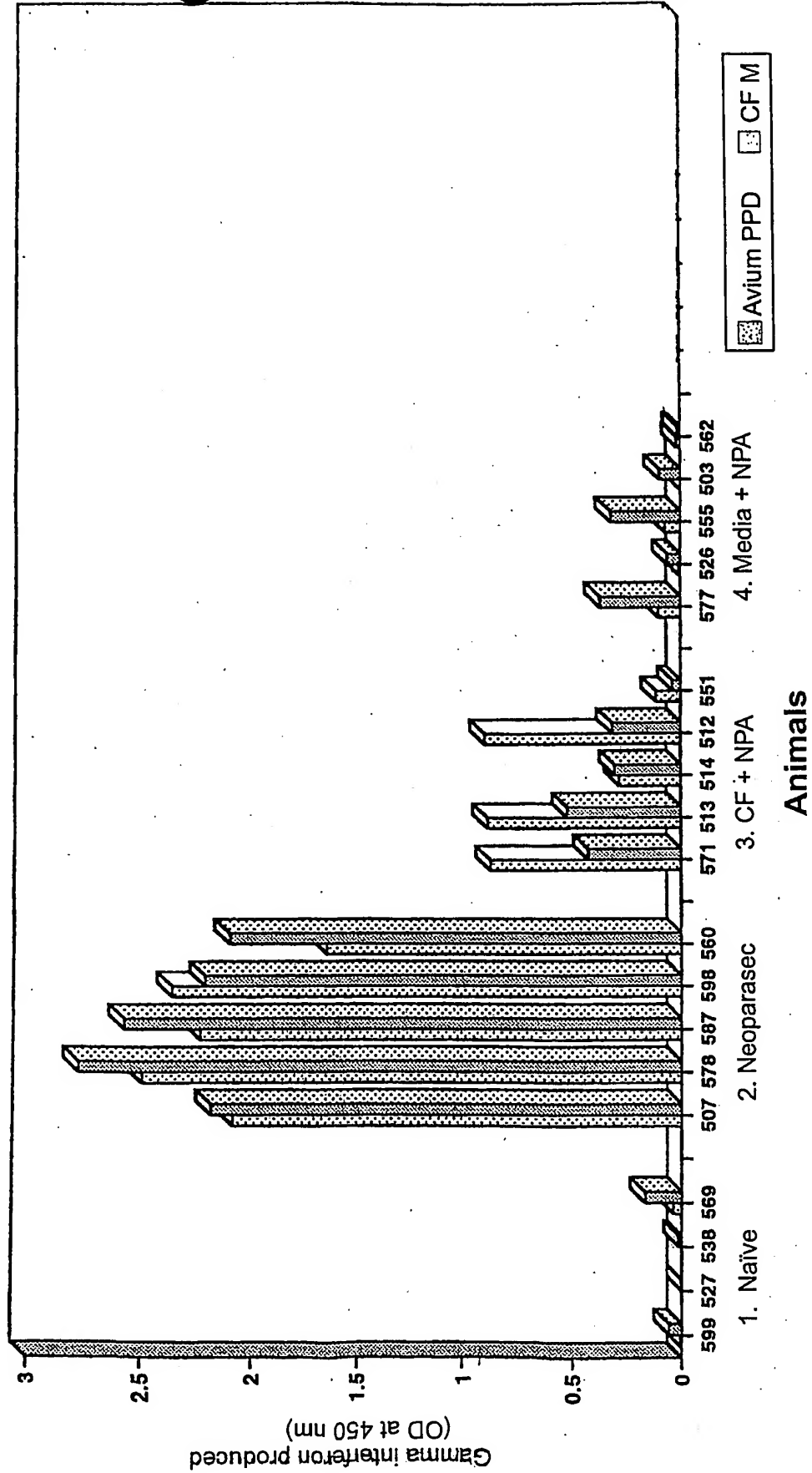


FIGURE 4

5/7

IFN- γ 6 mo post vaccination

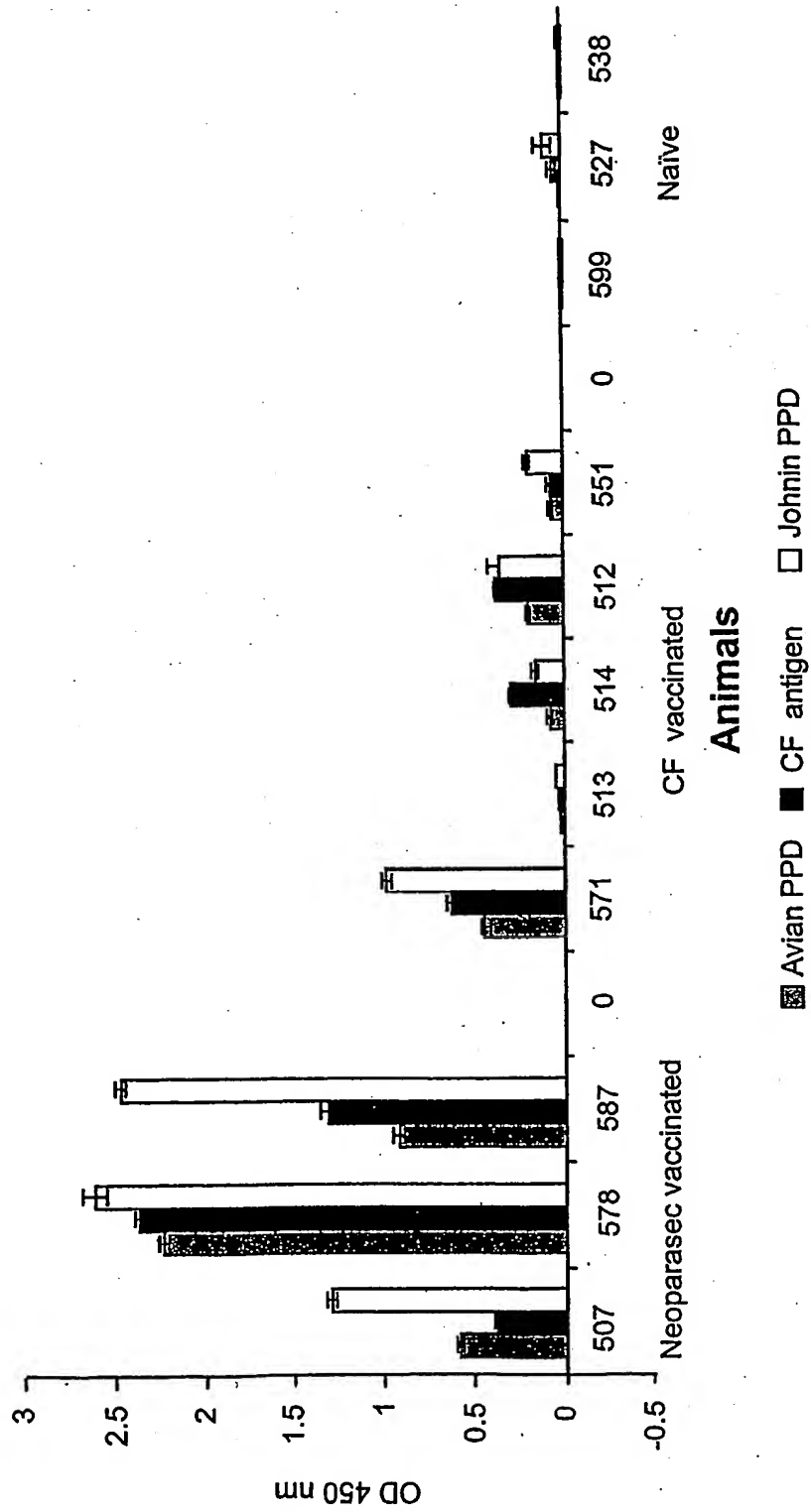


FIGURE 5

6/7

Mean IFN- γ response to avian PPD over time

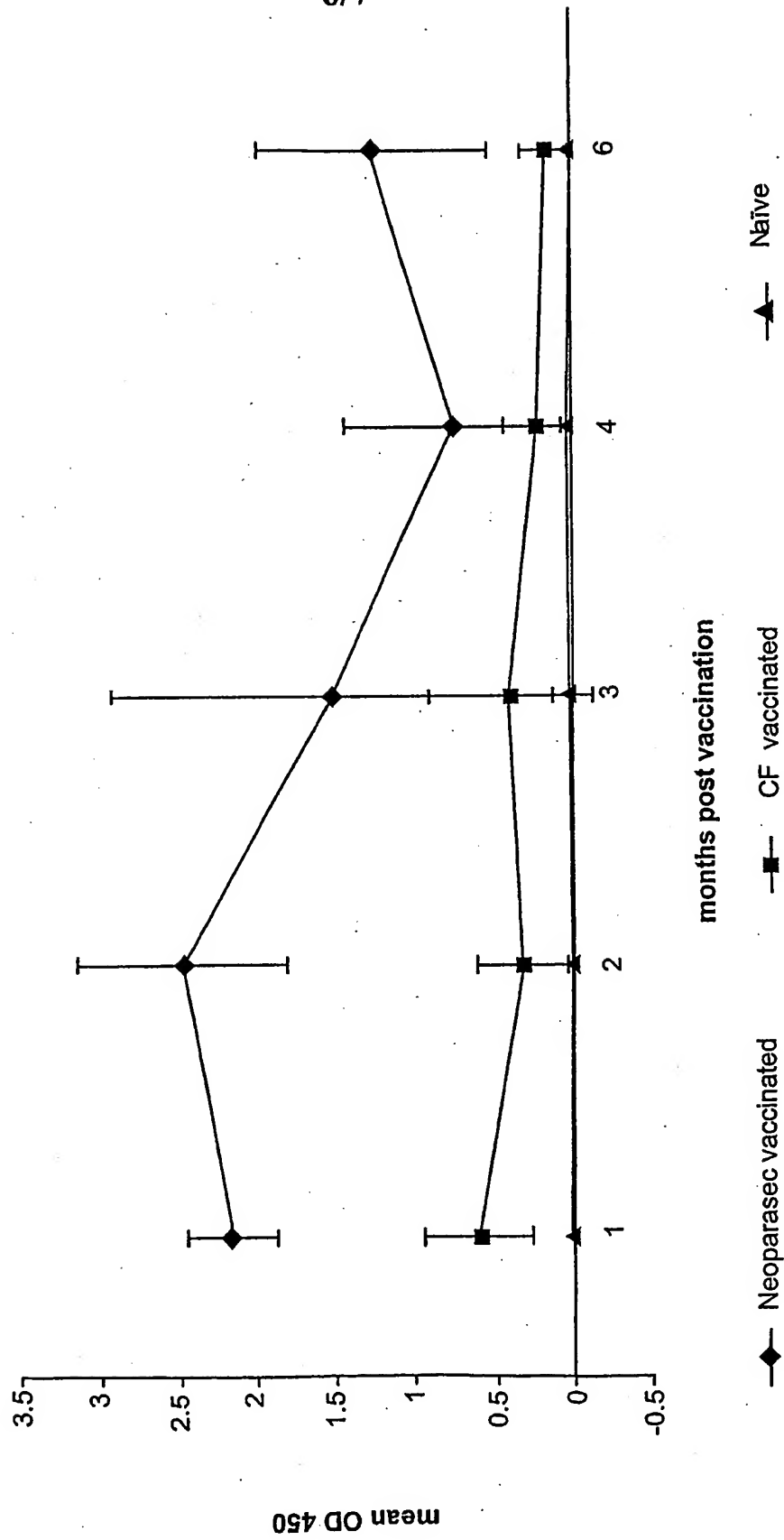


FIGURE 6

7/7

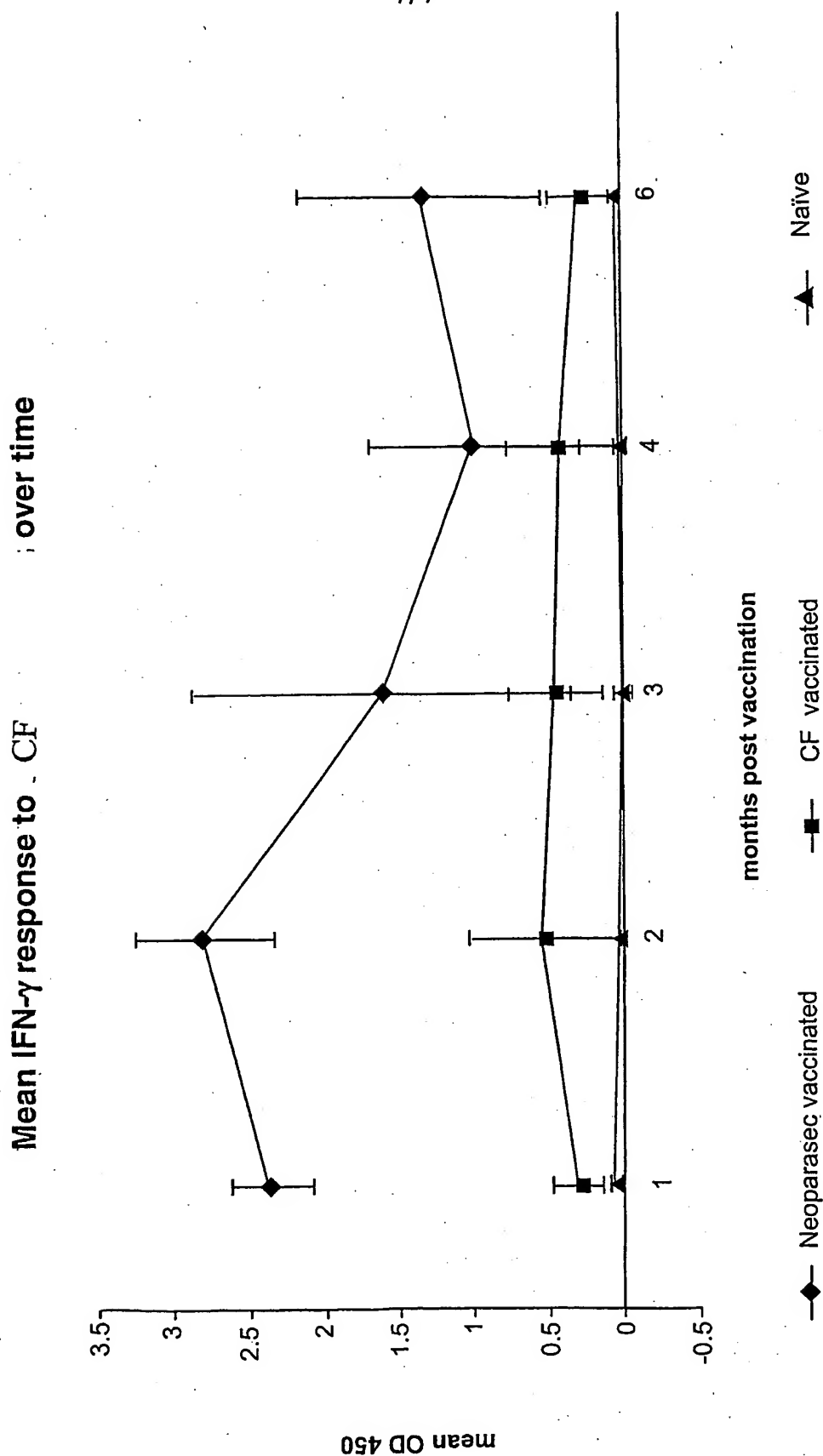


FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ02/00152

A. CLASSIFICATION OF SUBJECT MATTER		
Int. Cl. ⁷ : A61K 39/04; A61P 31/04, 31/06		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) DWPI and MEDLINE. Keywords: Mycobacterium avium, M.ptb, paratuberculosis, Johne's disease, Weybridge, vaccine and related terms.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6387372 B1 (COCITO et al.), 14 May 2002 Col. 1 lines 4-18, col.14 lines 3-12	1-12
X	US 6277580 B1 (ELLINGSON et al.), 21 August 2001 Claims 1 and 2	1-12
X	DE 19728834 A (GERLACH G), 7 January 1999 Whole document	1-12
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the international search 1 November 2002		Date of mailing of the international search report 14 NOV 2002
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer S. Chew Telephone No : (02) 6283 2248

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Derwent Abstract Accession No. 90-171668/23, Class B04 D16, BE 1002-022 A (ANDA BIOLOGICALS) 22 May 1990 Abstract	1-12
X	Valentin-Weigand P et al. Protein Antigens Secreted by Mycobacterium paratuberculosis, J. Vet. Med. B 1992, vol. 39, pages 762-766 Abstract	1-12
A	Derwent Abstract Accession No. 2002-247253/30, Class B04 D16, JP 2001342147 A (MOMOTANI E) 11 December 2001 Abstract	1-12

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
US	6387372	AU	14410/92	EP	577666
		WO	9216628	IL	101359
US	6277580	AU	47265/99	WO	2000/00505
DE	19728834	AU	88552/98	WO	99/02661
END OF ANNEX					

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.